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Simultaneous measurement of six cytokines in a single sample of human tears using microparticle-based flow cytometry: allergics vs. non-allergics

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Abstract

Tears play an essential role in maintaining corneal and conjunctival integrity by providing a tightly regulated, optimal extracellular environment critical to its numerous functions, which include anti-microbial defense, wound healing and inflammatory responses such as allergies. Elevated levels of inflammatory cytokines have been reported in tears from various ocular disease states. Characterization of tear cytokines has been limited by the small volume (microliter amounts) attainable. This limitation was addressed with the newly developed Becton Dickinson Cytometric Bead Array (CBA), which combines the principles of the "sandwich" immunoassay with the capability of flow cytometry for simultaneous measurement of the characteristics of multiple particles. This technique allows determination of six human cytokine (IFNγ, TNFα, IL-2, IL-4, IL-5, IL-10) concentrations simultaneously in a single tear sample. Tears were collected from the inferior fornix of non-allergic ($n = 7$) and allergic ($n = 9$) donors. Each tear sample or cytokine standard was incubated with a mixture of capture Ab-bead reagent and detector Ab-phycoerythrin (PE) reagent, and analyzed using flow cytometry. All six cytokines were detectable in both non-allergic and allergic tears. Tears from allergic donors contained significantly less IL-10 ($p = 0.035$), and had significant increases in the ratios of TNFα/IFNγ, IL-5/IFNγ and IL-5/IL-10 ($p = 0.0008$, 0.0124 and 0.011, respectively). The small volume required (5–10 μl/test) by the Cytometric Bead Array allows measurement of all six cytokines from a single collection of tears. This decreases collection time, minimizing the confounding effect of stimulation on cytokine concentration in tears, as well as allowing calculation of cytokine ratios. © 2001 Published by Elsevier Science B.V.

Keywords: Tears; Flow cytometry; Cytometric bead array; Cytokines; Allergy

Abbreviations: IFN, interferon; TNF, tumor necrosis factor; IL, interleukin; ELISA, enzyme-linked immunosorbent assay; TGF, transforming growth factor; VEGF, vascular endothelial growth factor; GM-CSF, granulocyte macrophage colony stimulating factor; SAC, seasonal allergic conjunctivitis; VKC, vernal keratoconjunctivitis; AKC, atopic keratoconjunctivitis; Ab, antibody; PE, phycoerythrin; FACS, fluorescence-activated cell sorter; CBA, cytometric bead array; BDIS, Becton Dickinson Immunocytometry Systems; MFI, mean fluorescence intensity; TH, T-helper

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1. Introduction

Tear film is a multi-functional interface between the external environment and the ocular surface. It is composed of three layers (mucous, aqueous and lipid), each secreted by a different set of orbital glands. These layers are functional components of the mucosal immune network, contributing to barrier defense at the ocular surface (Holly and Lemp, 1977). Tear film plays an essential role in maintaining corneal and conjunctival integrity by providing a tightly regulated, optimal extracellular environment critical to its numerous functions, including inflammatory responses such as allergies (Kijlstra and Kuizenga, 1994).

With recent advances in knowledge concerning the complex mechanisms of the cytokine network, researchers in ocular immunology have been turning their attention to the cytokine content of tears and its many implications in the processes of ocular inflammation. Inflammatory cytokines have been measured in normal tears including, IL-1α, IL-1β, IL-6, IL-8 (Nakamura et al., 1998, using pooled samples), TGF-β-1 and VEGF (Vesaluoma et al., 1997). Indeed, elevated levels of these cytokines (as well as the presence of TNFα, IL-4, IL-5, IFNγ, GM-CSF and eotaxin) have been reported in tears from various ocular allergic disease states such as seasonal allergic conjunctivitis (SAC) (Fujishima et al., 1995; Vesaluoma et al., 1999; Uchio et al., 2000), vernal keratoconjunctivitis (VKC) (Fujishima et al., 1995; Leonardi et al., 1998; Uchio et al., 2000), and atopic keratoconjunctivitis (AKC) (Fukagawa et al., 1999; Uchio et al., 2000). While these studies indicate that the ability to quantitate tear cytokines can increase our understanding of the pathology of ocular disease states, they also illustrate, due to the small volume of tears collectable, the necessity for pooling samples, measuring different cytokines from different donors, or painstakingly collecting the volume of tears necessary for multiple assays.

To overcome the limitations imposed by small sample volume, the Becton Dickinson Cytometric Bead Array (CBA) (Chen et al., 1999) was utilized in these studies. This newly developed technique uses uniform-size microparticle-based flow cytometry to measure a panel of six human cytokines (IFNγ, TNFα, IL-2, IL-4, IL-5, IL-10) simultaneously in a single tear sample. The ability of the CBA to measure the concentrations of six cytokines from the same tear sample permits calculation of cytokine ratios. Cytokine ratios are considered markers of various disease states such as asthma and atopy (Maggi, 1998; Kenyon et al., 2000). The relative balance between various cytokines is often considered more important than the absolute concentrations of cytokines in an individual.

2. Materials and methods

2.1. Human donors

Sixteen donors were recruited for the study, eleven women and five men, between the ages of 20 and 50. The protocol was approved by the Health Sciences Human Subjects Committee of the University of Wisconsin-Madison, and written informed consent was obtained from all participants.

2.2. Skin tests

Donors were screened for allergies using a skin prick-puncture test (Greer Laboratories, Lenoir, NC) with histamine as a positive control, and the diluent fluid as a negative control. Donors reacting only to histamine were classified as non-allergics ($n = 7$). Donors reacting to allergens were classified as allergic donors ($n = 9$). Allergic donors responding only to seasonal allergens (i.e. ragweed, grass, tree pollens) were further classified as seasonal allergics ($n = 3$). Allergic donors responding to perennial allergens (i.e. house dust mite, cat, dog) were further classified as perennial allergics (even if they responded to seasonal allergens as well, $n = 6$).

2.3. Sample collection

Tears were collected from all donors between 8:00 AM and 12:00 noon during January and February. A 5-μl glass microcapillary pipette was atraumatically placed just inside the lateral canthal margin to collect tears from the inferior fornix. Tears were repeatedly expelled into 0.5-ml microcentrifuge tubes

from the microcapillary pipette, until sufficient volume (~5–15 μl) was acquired. None of the donors appeared to have an active ocular allergic response occurring at the time of tear collection.

2.4. Cytometric bead array immunoassay

Fig. 1 is a diagram of the Becton Dickinson Cytometric Bead Array. Microparticles were prepared and coupled to antibodies (Ab) as previously described (Chen et al., 1999). Briefly, particles (polystyrene beads, 7.5 μm, Bang's Laboratories) are dyed to six different fluorescence intensities. The proprietary dye has an emission wavelength of ~650 nm (FL-3). Each particle is coupled via a covalent linkage based on thiol-maleimide chemistry with an Ab (PharMingen, San Diego, CA) against one of six cytokines (IFNγ, TNFα, IL-2, IL-4, IL-5, IL-10) and represents a discrete population, unique in their FL-3 intensity. The Ab-particles serve as capture for a given cytokine in the immunoassay panel and can be detected simultaneously in a mixture. The captured cytokines are detected via direct immunoassay using six different antibodies coupled to phycoerythrin (PE), which emits at ~585 nm (FL-2). The calibrators (standards ranging from 0 to 5000 pg/ml) for the assay system (capture Ab-bead reagent, detector Ab–PE reagent) are mixtures of all six cytokines. Six standard curves are thus obtainable from one set of calibrators (six results obtained from each calibration point shown in Fig. 2).

For each tear sample and cytokine standard mixture, 5–10 μl of sample or standard was added to a mixture of 50 μl each of capture Ab-bead reagent and detector Ab–PE reagent. The mixture (105–110 μl) was subsequently incubated for 20 h at room temperature, and washed to remove unbound detector Ab–PE reagent before data acquisition using flow cytometry.

2.5. Recovery experiments

Spike-recovery experiments were conducted using pooled tears from five donors to verify that the assay could measure cytokines added to the normal tear matrix with acceptable recovery. Cytokine spike concentrations were 80, 625 and 2500 pg/ml. The spiking volume was 5% of the sample (i.e. tear volume is 95%). A mixture of 50 μl each of capture Ab-bead reagent and detector Ab–PE reagent was added to 10 μl of pooled spiked sample or calibrator, and incubated for 20 h at room temperature prior to washing and data acquisition using flow cytometry.

2.6. Flow cytometric analysis

Two-color flow cytometric analysis was performed using a FACScan® or a FACSCalibur® flow cytometer [Becton Dickinson Immunocytometry Systems (BDIS), San Jose, CA]. Data were acquired and analyzed using Becton Dickinson (BD) Cytometric Bead Array (CBA) software. Forward vs. side scatter gating was employed to exclude any sample particles other than the 7.5-μm polystyrene beads. Data were displayed as two-color dot plots (FL-2 vs. FL-3) such that the six discrete FL-3 microparticle dye intensities were distributed along the *Y*-axis. Standard curves were plotted [cytokine calibrator concentration vs. FL-2 mean fluorescence intensity (MFI)] using a four-parameter logistic curve fitting model. Cytokine concentrations were determined from these standard curves. If a sample had a cytokine concentration below the detection limit for the assay, a value of 0 was assigned for that particular cytokine concentration.

Cytokine (TNFα, IL-2, IL-4, IL-5 and IL-10)/IFNγ ratios were calculated to examine the TH_2 vs. TH_1 balance of cytokines. Cytokine (TNFα, IL-2, IL-4, IL-5 and IFNγ)/IL-10 ratios were also calculated. If the cytokine concentration used in the denominator was 0 (undetectable), the ratio was eliminated from the analysis.

2.7. Statistical analyses

Data was analyzed using SAS® software (SAS Institute, Cary, NC). A general linear-model analysis of variance (ANOVA) with pre-planned comparison was computed to generate two-tailed *p*-values. Fisher least significant-difference tests were used to make appropriate post ANOVA comparisons. Probabilities ≤ 0.05 were considered statistically significant. The primary aims of this study were method development

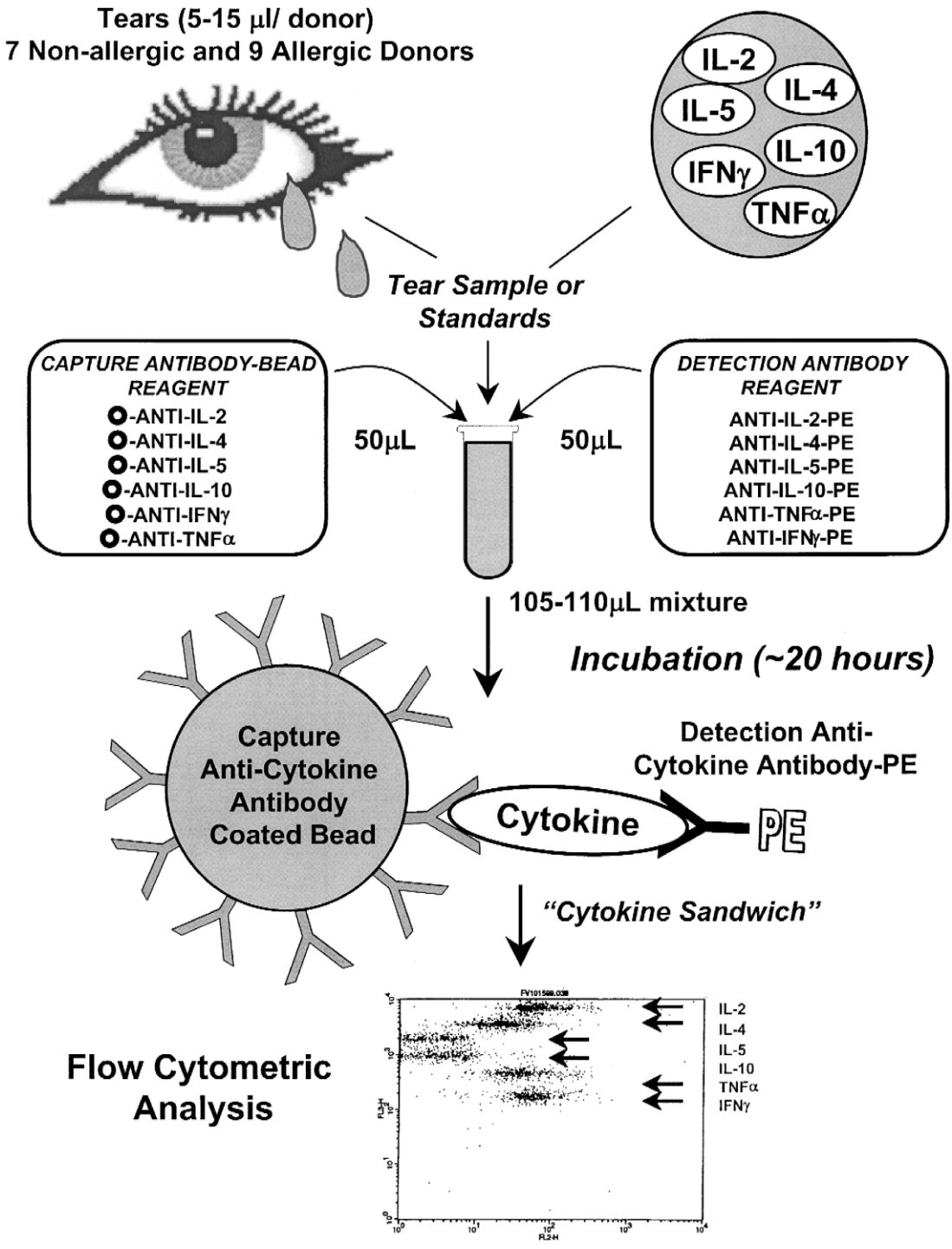

Fig. 1. A schematic of the Cytometric Bead Array immunoassay procedure. Beads of different fluorescence intensity, coated with different cytokine antibodies and detector cytokine antibodies coupled to phycoerythrin (PE), are incubated with sample or standards for 20 h and analyzed by flow cytometry.

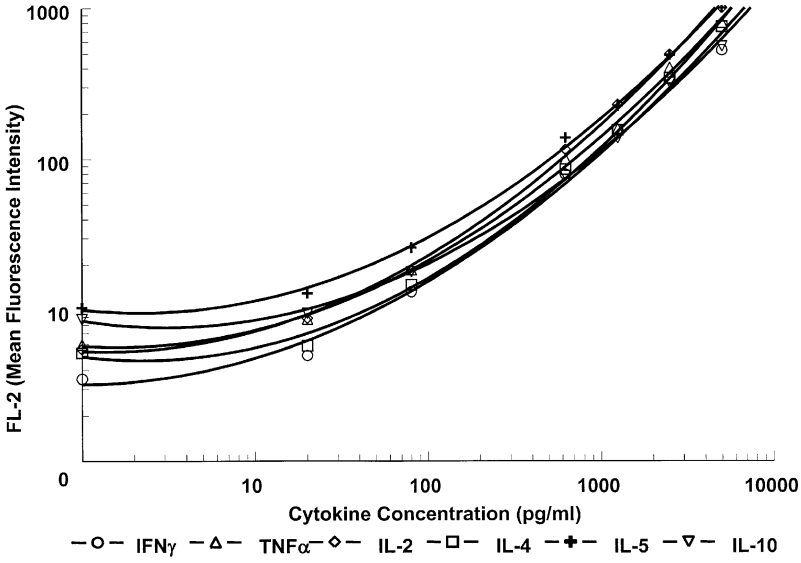

Fig. 2. Representative standard curves for IFNγ, TNFα, IL-2, IL-4, IL-5, and IL-10.

and potential applications for research; therefore probabilities ≤ 0.1 are also discussed. All data are presented as mean $\pm$ S.E.M.

3. Results

3.1. Standard curves and spike recovery

The performance of the CBA for all six cytokines was previously evaluated by BDIS and PharMingen using cell culture supernatants, human sera and plasma (data not shown). Analytical sensitivity was comparable to conventional ELISA (correlation coefficients $> 90\%$) and detection limits ranged from 2 to 20 pg/ml. The standard curves are shown in Fig. 2. Representative dot plots of the standards vs. spiked tears for each cytokine are shown in Fig. 3. Spike recovery experiments illustrated the consistencies of recovery throughout the range of calibration in the normal tear matrix. The 80 pg/ml spike was not statistically distinguishable from the zero spike due to the high concentrations of all six cytokines in the pooled tears (250.6 ± 30.9 pg/ml). Therefore, the mean percent recoveries calculated for each cytokine were based on the 625 and 2500 pg/ml spikes and were as follows: IFNγ, 67 ± 3; TNFα, 43 ± 8; IL-2, 64 ± 6; IL-4, 66 ± 4; IL-5, 86 ± 5; IL-10, 63.5 ± 7.5.

3.2. Tear cytokine evaluation

All six cytokines were detectable in tears from both non-allergic and allergic donors. The concentrations, in pg/ml, are shown in Fig. 4. Tears from allergic donors had significantly lower IL-10 concentrations ($p = 0.035$), with a trend toward decreased IFNγ concentrations ($p = 0.137$), compared to tears from non-allergic donors.

TNFα, IL-2, IL-4, IL-5, and 10/IFNγ ratios are shown in Fig. 5. There was a highly significant increase in the ratios of TNFα/ IFNγ and IL-5/IFNγ in tears from allergic donors ($p = 0.0008$ and 0.0124, respectively) accompanied by a trend toward a decrease in the ratio of IL-10/IFNγ ($p = 0.115$).

TNFα, IL-2, IL-4, IL-5 and IFNγ/IL-10 ratios were also calculated, and shown in Fig. 6. In tears from allergic donors, the IL-5/IL-10 ratio was significantly increased ($p = 0.011$), and a trend toward increased TNFα/IL-10, IFNγ/IL-10, IL-2/IL-10 and IL-5/IL-10 ratios was observed ($p = 0.062$, 0.173, 0.140 and 0.095, respectively).

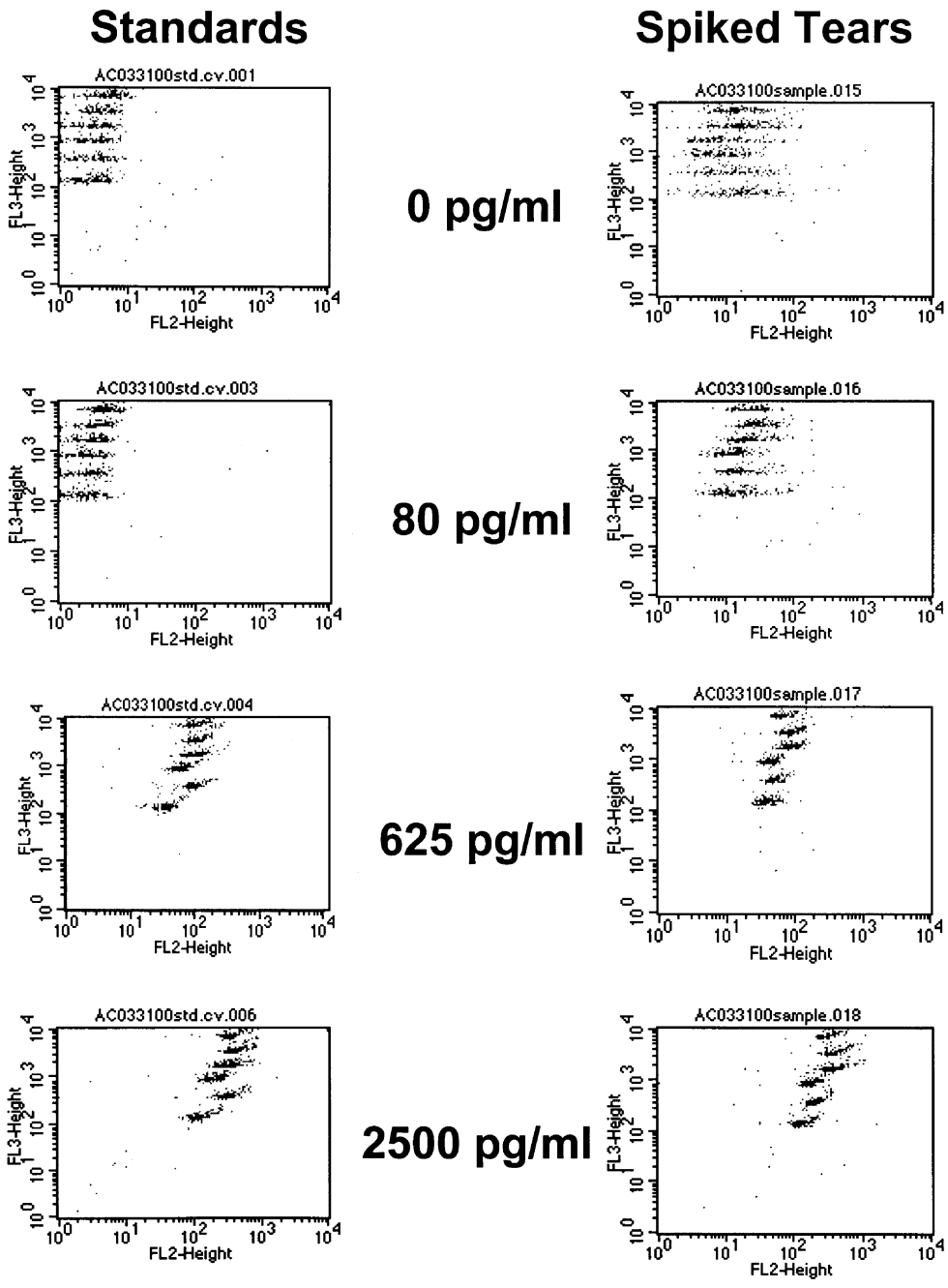

Fig. 3. Representative dot plots of cytokine standards vs. pooled non-allergic tears spiked with cytokine standards.

No differences in cytokine concentrations or ratios between perennial and seasonal allergic donors were found (data not shown). However, the patterns of ratios observed (i.e. increased TNFα/IFNγ,

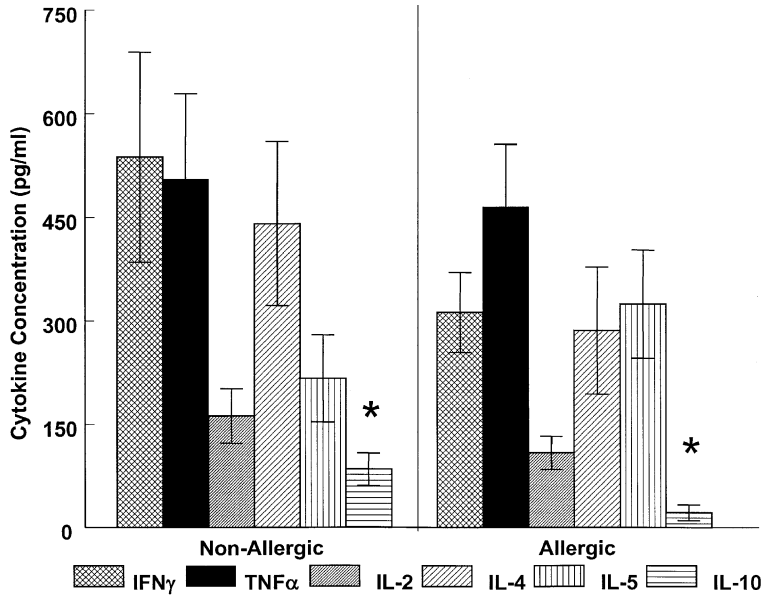

Fig. 4. Concentrations of cytokines measured in non-allergic ($n = 7$) vs. allergic ($n = 9$) donor tears (mean ± standard error, * concentrations are significantly different from each other, $p < 0.05$).

IL-5/IFNγ and IL-5/IL-10 ratios in tears from allergic donors compared to tears from non-allergic donors) were similar for both groups of allergic donors, even though it was doubtful that the seasonal

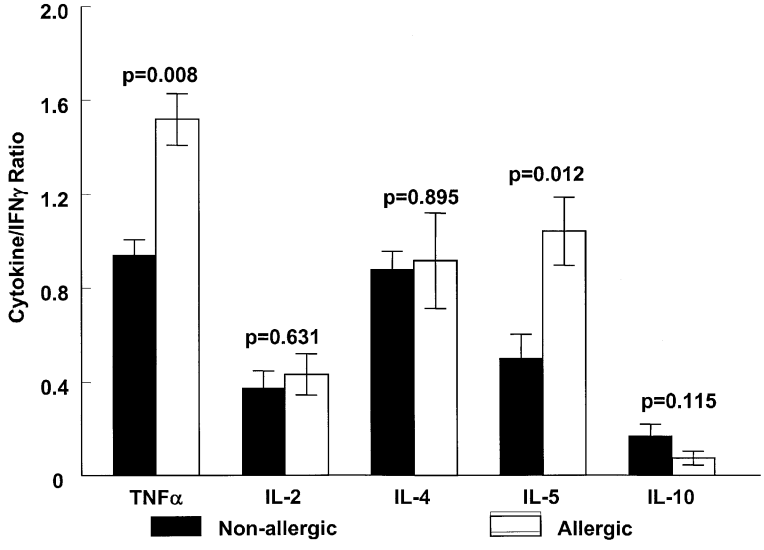

Fig. 5. Ratios of cytokine concentration/IFNγ concentration in non-allergic ($n = 7$) vs. allergic ($n = 9$) donor tears (mean ± standard error, differences of $p < 0.05$ are statistically significant).

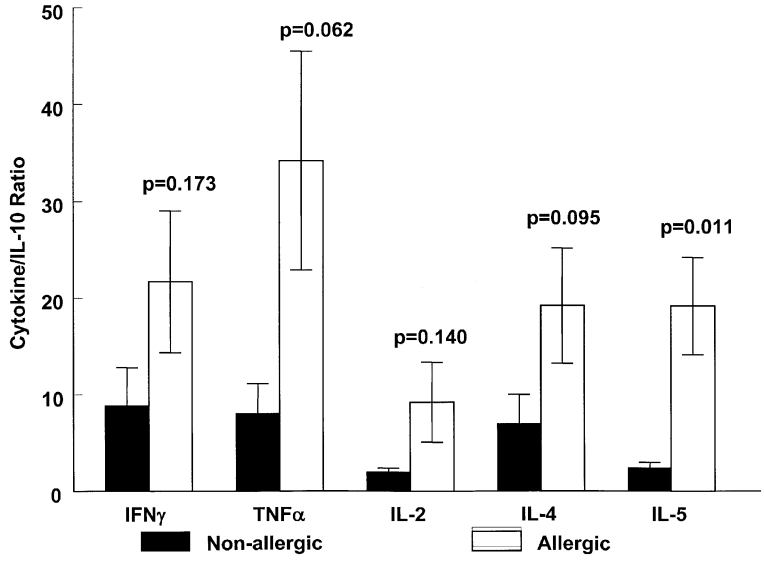

Fig. 6. Ratios of cytokine concentration/IL-10 concentration in non-allergic ($n = 7$) vs. allergic ($n = 9$) donor tears (mean ± standard error, differences of $p < 0.05$ are statistically significant).

allergic donors had recently been exposed to their respective allergens in January or February.

4. Discussion

We have demonstrated the ability to simultaneously measure six cytokine concentrations from a single sample of human tears using the Becton Dickinson Cytometric Bead Array. Our results agree with previously reported cytokine concentrations in human tears (Nakamura et al., 1998; Uchio et al., 2000). This microparticle-based flow cytometric immunoassay has been proven in comparison studies to have comparable analytical sensitivity to conventional ELISAs (Chen et al., 1999). Insufficient tear sample volume prevented comparing ELISAs. Accordingly, recovery experiments were performed using tear samples spiked with recombinant cytokines to confirm the ability of the assay to measure cytokines added to the normal tear matrix. Recovery experiments are important because various proteins (i.e. soluble receptors, degradation enzymes) are conceivably present in tears and may interfere with the assessment of cytokine concentrations. Although we are not aware of any reports in the literature, this may explain, in particular, the lower recovery of TNFα. It is important to point out that the spike recovery experiments did show consistencies of recovery throughout the range of calibration in the normal tear matrix, and recoveries of recombinant cytokines from spiked tears were similar to recoveries of recombinant cytokines from spiked serum (Chen et al., 1999).

It is well documented that protein concentrations present in basal tears are different from stimulated tears. When the eye is touched mechanically, stimulation occurs, therefore, basal tears must either be obtained through application of a filter to the eye or needle aspiration. With the capillary technique used in this study, the tears collected would be defined as stimulated tears. Several researchers, using a variety of techniques, have demonstrated that concentrations of serum proteins including albumin, transferrin (Stuchell et al., 1984), immunoglobulins (Fullard and Snyder, 1990), and cytokines (Nakamura et al., 1998) are lower in stimulated tears presumably due to dilution. Consequently, tear cytokine protein concentrations are sensitive to collection technique. As previously mentioned, an important feature of the CBA analysis of tears is that it allows for the calculation of cytokine ratios. These ratios, while providing important information as markers for certain disease states, also allow a better comparison of cytokine data between samples. Given the complexi-

ties of the cytokine network, the relative balance of cytokines may better reflect the net effect of cytokine responses than the absolute concentrations of cytokines. Cytokine ratios (i.e. TH_2 vs. TH_1 or pro-inflammatory vs. anti-inflammatory) have been used to characterize a variety of disease states (Hagiwara et al., 1996; Othoro et al., 1999; Moverare et al., 2000; Kenyon et al., 2000).

Since IL-10 is an anti-inflammatory cytokine, it is particularly interesting that IL-10 concentrations were significantly lower in tears from allergic donors tears compared to tears from non-allergic donors. IL-10 has been shown to downregulate antigen-dependent activation of cells involved in allergic reactions and prevent eosinophil homing to inflammatory sites in experimental models of allergic inflammation (Pretolani, 1999). Reduced levels of IL-10 have been reported in bronchoalveolar lavage fluid and peripheral blood mononuclear cells from asthmatic patients (Borish et al., 1996). The reduced level of IL-10 in the tears of allergic donors results in a trend toward increased tear pro-inflammatory cytokines/IL-10 ratios. The reduced level of IL-10 in the tears of allergic donors could be an important attribute of the pathophysiology of ocular allergic inflammation.

Even more striking were the significantly increased TNFα/ IFNγ and IL-5/IFNγ ratios in tears from allergic donors (resulting from a combination of higher concentrations of TNFα and IL-5 and lower concentrations of IFNγ). TNFα is a pro-inflammatory cytokine that increases in tears following ocular allergen challenge (Vesaluoma et al., 1999). We have characterized the critical role TNFα plays in conjunctival epithelial cell ICAM-1 upregulation using an in vitro model for studying interactions between human conjunctival mast cells and epithelial cells (Cook et al., 1998, 2000). An increased IL-5/IFNγ ratio is a feature of allergic diseases (and thus, the TH_2 phenotype). It is generally accepted that IL-5 is required to generate an eosinophilic response in allergic disease. Little is known, however, about the importance of IL-5 in ocular allergic inflammation. A recent study demonstrated that increased IL-5 levels in tears correlated with proliferative lesions in VKC and AKC (Uchio et al., 2000). Eosinophil-derived mediators likely contribute to the corneal tissue destruction that ultimately leads to the impairment of vision in AKC and VKC.

We believe the CBA technique can make a valuable contribution in understanding the specific immunopathologic mechanisms underlying cytokine interaction with the ocular surface. Although our data from a relatively small number of donors could be considered preliminary, nevertheless, it establishes the usefulness of this technique. Characterization of cytokine profiles in ocular allergic disease states could provide an important key to discerning why certain allergic individuals have self-limited, mild and seasonal disease, while others go on to have a sustained and significant immunopathologic reaction leading to sight-threatening disease.

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